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(57) Abstract

Enzymatic RNA molecules which cleave apo(a) mRNA. Use of these catalytic RNA molecules for the treatment of conditions related to lipoprotein A levels, such as atherosclerosis, myocardial infarction, strokes; testenosis and heart diseases.

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DESCRIPTION

RIBOZYME TREATMENT OF DISEASES OR CONDITIONS RELATED TO LEVELS OF PLASMA LIPOPROTEIN (a) [Lp(a)] BY INHIBITING APOLIPOPROTEIN (a) [APO(a)]

Field of the Invention

The present invention relates to therapeutic compositions and methods for the treatment or diagnosis of diseases or conditions related to Lp(a) levels, such as atherosclerosis, myocardial infarction, stroke, and restenosis.

Background Of The Invention

The following is a brief description of the physiological role of Lp(a). The discussion is not meant to be complete and is provided only for understanding of the invention that follows. This summary is not an admission that any of the work described below is prior art to the claimed invention.

Low density lipoproteins (LDLs) are mainly composed of cholesterol, phospholipids and a single hydrophobic protein, apolipoprotein B [apoB]. They are considered as the major carriers of cholesterol in human plasma (for review see Uterman, G. (1989) Science 246, 904-910). ApoB, the only protein subunit of LDL, recognizes and binds to LDL receptors on the surface of cells. This LDL-LDL receptor interaction results in the internalization of the LDL and the eventual release of cholesterol inside the cell.

A modified form of LDL, termed as lipoprotein (a) [Lp(a)], was discovered in 1963 [Berg, K '1963) Acta Pathol. Microbiol. Scand. 59, 369]. Covalent linkage of an additional glycoprotein, apo(a), to the LDL distinguishes Lp(a) from LDL. Several studies have recently suggested that elevated levels of Lp(a) in human plasma is linked to heart disease (Gurakar, et al., (1985) Atherosclerosis 57, 293-301; Leren, et al., (1988) Atherosclerosis 73, 135-141; Utermann, Supra). The Lp(a) levels range over 1000 fold and individuals with top quartile of plasma Lp(a) levels have two-to five-fold increased probability of developing atherosclerosis.

Atherosclerosis is a disease associated with hardening and loss of elasticity of arterial walls. High concentrations of cholesterol, in the form of Lp(a), in human blood plasma is one of the most important factors responsible for atherosclerosis. Deposition of cholesterol in the Macrophages and smooth muscle cells associated with arterial walls cause plaques (atheromatous lesions) which cause proliferation of adjoining smooth muscle cells. With time, these plaques grow in size causing hardening of the arterial walls and loss of elasticity, which in turn results in rupturing of the arterial walls, blood clotting and blockage of blood flow in the artery (for datails see Textbook of medical physiology Guyton, A.C., (Saunders Company, Philadelphia, 1991) pp. 761-764).

Lp(a) and/or apo(a) levels correlate well with an increased risk of atherosclerosis and its subsequent manifestations such as myocardial infarction, stroke, and restenosis. The apo(a) protein is unique to humans, Old World primates and hedgehogs; its absence in common laboratory animals has made exploration of the physiological role of apo(a) levels difficult. Recently, a transgenic mouse expressing the human gene encoding apo(a) was constructed [Lawn et al., (1992) Nature 360, 670-672]. The transgenic mice are more susceptible than control liter-mates to the development of lipid-rich regions in the aorta. Moreover, human apo(a) expression colocalizes to the regions of fat deposition. Thus, overexpression of apo(a) directly leads to atherosclerotic-like lesions in experimental animals. This observation lends credence to the hypothesis that elevated levels of apo(a) in humans contribute to atherosclerotic disease.

Apolipoprotein(a) is a large glycoprotein which varies in size from 300-800 KDa. Thirty four different isoforms have been characterized from human plasma. The only human cDNA clone currently available encompass 14 kilobase message that encodes apo(a) [McLean et al., (1987) Nature 330, 132-137]. A Rhesus monkey cDNA representing a part of the 3' end of the apo(a) mRNA has also been cloned and sequenced (Tomlinson et al., 1989 J. Biol. Chem. 264, 5957-5965). Sequence analysis of the cloned cDNA revealed two unique facets of the apo(a) structure. First, the apo(a) cDNA is remarkably repetitious. The reconstructed apo(a) cDNA encodes a protein of 4,529 amino acids; 4,210 of the residues are present in 37 repeats of 114 amino acids each. The

repeated units themselves are especially homologous; 24 are identical in nucleotide sequence, four more share a sequence that differs in only three nucleotides and the remaining repeats differ by only 11 to 71 bases.

Secondly, apolipoprotein(a) is highly homologous to the serine protease, plasminogen. Plasminogen consists of five repeated homologous domains termed kringles (which are approximately 50% homologous in their amino sequences) followed by a trypsin-like protease domain. Kringle IV of plasminogen is very homologous to the 37 repeats of apo(a) [75-85% at the protein level). In addition, the 5′ untranslated region, the signal peptide region, kringle V, the protease domain, and the 3′ untranslated region of plasminogen are 98%, 100%, 91%, 94% and 87% homologous to apo(a) sequences, respectively. Relative to plasminogen, apo(a) is missing kringles I, II, and III and, as mentioned above, has extensively duplicated kringle IV. Despite the high degree of homology apo(a) cannot be converted into a protease by tissue type plasminogen activator (tPA). This is because of a single amino acid substitution in apo(a) at the site of activation of plasminogen by tPA (Utermann, *supra*). *IN vitro* studies have indicated that apo(a) and Lp(a) compete with plasminogen for binding to the plasminogen receptor and fibrin which supports the correlation between high Lp(a) levels and myocardial infraction (Gonzalez-Gronow et al., (1989) Biochemistry 28, 2374-2378; Hajjar et al., (1989) Nature 339, 303-305; Miles et al., (1989) Nature 339, 301-303). Recent *in vivo* studies in human (Moliterno et al., 1993 Circulation 88, 935-940) and monkey (Williams et al., 1993 Atheroscler. Thromb. 13, 548-554) support a role for Lp(a) in preventing clot lysis.

The extraordinary homology between apo(a) and plasminogen presents several barriers to drug development. Small molecule inhibitors of apo(a) would have to selectively bind apo(a) without negatively impacting plasminogen function. Similarly, antisense approaches will be limited by the overall nucleotide sequence homology between the two genes. Current dietary and drug therapies (Gurakar, *et al., supra*; Leren *et al., supra*), with the exception of nicotinic acid, have little or no effect on apo(a) levels.

Applicant now shows that these same limitations are opportunities for ribozyme therapy. The cleavage site specificity of ribozymes allows one to

identify ribozyme target sites present in apo(a) mRNA but completely absent in the mRNA of plasminogen. For instance, there are 13 hammerhead cleavage sites present in the highly conserved kringles of apo(a) that are not present in kringle IV of plasminogen. Likewise, the last kringle repeat, protease domain and 3′ untranslated region of apo(a) contain 21 hammerhead ribozyme cleavage sites present in apo(a) that are not present in plasminogen. Thus, ribozymes that target apo(a) mRNA represent unique therapeutics and diagnostic tools for the treatment and diagnosis of those at high risk of atherosclerosis.

Summary of the Invention

This invention relates to ribozymes, or enzymatic RNA molecules, directed to cleave mRNA species encoding apo(a). In particular, applicant describes the selection and function of ribozymes capable of cleaving this RNA and their use to reduce levels of apo(a) in various tissues to treat the diseases discussed herein. Such ribozymes are also useful for diagnostic uses.

Ribozymes that cleave apo(a) mRNA represent a novel therapeutic approach to atherosclerosis. Ribozymes may show greater perdurance or lower effective doses than antisense molecules due to their catalytic properties and their inherent secondary and tertiary structures. Such ribozymes, with their catalytic activity and increased site specificity (as described below), represent more potent and safe therapeutic molecules than antisense oligonucleotides.

Applicant indicates that these ribozymes are able to inhibit expression of apo(a) and that the catalytic activity of the ribozymes is required for their inhibitory effect. Those of ordinary skill in the art, will find that it is clear from the examples described that other ribozymes that cleave target apo(a) encoding mRNAs may be readily designed and are within the invention.

Six basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. Table I summarizes some of the characteristics of these ribozymes. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a

enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over other technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its processing and translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cle many molecules of target RNA. In addition, the ribozyme is a highly s cific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf, T. M., et al., 1992, Proc. Natl. Acad. Sci. USA, 89, 7305-7309). Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

In preferred embodiments of this invention, the enzymatic nucleic acid molecule is formed in a hammerhead or hairpin motif, but may also be formed in the motif of a hepatitis delta virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or *Neurospora* VS RNA. Examples of such hammerhead motifs are described by Rossi *et al.*, 1992, Aids Research and Human Retroviruses, 8, 183, of hairpin motifs by Hampel *et al.*, "RNA Catalyst for Cleaving Specific RNA Sequences," filed September 20, 1989, which is a continuation-in-part of U.S. Serial No. 07/247,100 filed September 20, 1988, Hampel and Tritz, 1989, Biochemistry , 28, 4929, and Hampel *et al.*, 1990, Nucleic Acids Res. 18,299, and an example of the hepatitis delta virus motif is described by

Perrotta and Been, 1992, *Biochemistry* , 31, 16, of the RNaseP motif by Guerrier-Takada et al., 1983,*Cell*, 35, 849, *Neurospora* VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 61, 685-696; Saville and Collins, 1991 Proc. Natl. Acad. Sci. USA 88, 8826-8830; Collins and Olive, 1993 Biochemistry 32, 2795-2799) and of the Group I intron by Cech et al., U.S. Patent 4,987,071. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

The invention provides a method for producing a class of enzymatic cleaving agents which exhibit a high degree of specificity for the RNA of a desired target. The enzymatic nucleic acid molecule is preferably targeted to a highly conserved sequence region of a target apo(a) encoding mRNA such that specific treatment of a disease or condition can be provided with either one or several enzymatic nucleic acids. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells as required. Alternatively, the ribozymes can be expressed from DNA vectors that are delivered to specific cells.

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small enzymatic nucleic acid motifs (*e.g.*, of the hammerhead or the hairpin structure) are used for exogenous delivery. The simple structure of these molecules increases the ability of the enzymatic nucleic acid to invade targeted regions of the mRNA structure. However, these catalytic RNA molecules can also be expressed within cells from eukaryotic promoters (e.g., Scanlon, K. J., et al., 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet, M., et al., 1992, *Antisense Res. Dev.*, 2, 3-15; Dropulic, B., et al., 1992, *J Virol*, 66, 1432-41; Weerasinghe, M., et al., 1991, *J Virol*, 65, 5531-4; Ojwang, J. O., et al., 1992, *Proc. Natl. Acad. Sci. USA* , 89, 10802-6; Chen, C. J., et al., 1992, *Nucleic Acids Res.*, 20, 4581-9; Sarver, H., et al., 1990, *Science*, 247, 1222-1225)). Those skilled in the art realize that any ribozyme can be expressed in eukaryotic cells from the appropriate DNA vector. The activity

of such ribozymes can be augmented by their release from the primary transcript by a second ribozyme (Draper et al., PCT WO93/23569, and Sullivan et al., PCT WO94/02595, both hereby incorporated in their totality by reference herein; Ohkawa, J., et al., 1992, *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira, K., et al., 1991, *Nucleic Acids Res.*, 19, 5125-30; Ventura, M., et al., 1993, *Nucleic Acids Res.*, 21, 3249-55) .

Thus, in a first aspect, the invention features ribozymes that inhibit apo(a) production. These chemically or enzymatically synthesized RNA molecules contain substrate binding domains that bind to accessible regions of their target mRNAs. The RNA molecules also contain domains that catalyze the cleavage of RNA. The RNA molecules are preferably ribozymes of the hammerhead or hairpin motif. Upon binding, the ribozymes cleave the target apo(a) encoding mRNAs, preventing translation and protein accumulation. In the absence of the expression of the target gene, a therapeutic effect may be observed.

By "inhibit" is meant that the activity or level of apo(a) encoding mRNA is reduced below that observed in the absense of the ribozyme, and preferably is below that level observed in the presence of an inactive RNA molecule able to bind to the same site on the mRNA, but unable to cleave that RNA.

Such ribozymes are useful for the prevention of the diseases and conditions discussed above, and any other diseases or conditions that are related to the level of apo(a) activity in a cell or tissue. By "related" is meant that the inhibition of apo(a) mRNA translation, and thus reduction in the level of apo(a), will relieve to some extent the symptoms of the disease or condition.

Ribozymes are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells. The RNA or RNA complexes can be locally administered to relevant tissues through the use of a catheter, infusion pump or stent, with or without their incorporation in biopolymers. In preferred embodiments, the ribozymes have binding arms which are complementary to the sequences in Tables II, IV, VI and VII. Examples of such ribozymes are shown in Tables III, V, VI and VII Examples of such ribozymes consist essentially of sequences defined in these Tables. By "consists essentially of" is meant that the active

ribozyme contains an enzymatic center equivalent to those in the examples, and binding arms able to bind mRNA such that cleavage at the target site occurs. Other sequences may be present which do not interfere with such cleavage.

In another aspect of the invention, ribozymes that cleave target molecules and inhibit apo(a) activity are expressed from transcription units inserted into DNA, RNA, or viral vectors. Preferably, the recombinant vectors capable of expressing the ribozymes are locally delivered as described above, and transiently persist in target cells. Once expressed, the ribozymes cleave the target mRNA. The recombinant vectors are preferably DNA plasmids or adenovirus vectors. However, other mammalian cell vectors that direct the expression of RNA may be used for this purpose.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description Of The Preferred Embodiments

The drawings will first briefly be described.

Drawings:

Figure 1 is a diagrammatic representation of the hammerhead ribozyme domain known in the art. Stem II can be ≥ 2 base-pair long or may be a loop region without base pairing.

Figure 2a is a diagrammatic representation of the hammerhead ribozyme domain known in the art; Figure 2b is a diagrammatic representation of the hammerhead ribozyme as divided by Uhlenbeck (1987, *Nature*, 327, 596-600) into a substrate and enzyme portion; Figure 2c is a similar diagram showing the hammerhead divided by Haseloff and Gerlach (1988, *Nature* , 334, 585-591) into two portions; and Figure 2d is a similar diagram showing the hammerhead divided by Jeffries and Symons (1989, *Nucl. Acids. Res.*, 17, 1371-1371) into two portions.

Figure 3 is a representation of the general structure of the hairpin ribozyme domain known in the art. H, is A, U or C. Y is U or C. N is A, U, G,

or C. N' is the complementary sequence of N. Helix 4 can be ≥ 2 base-pair long

Figure 4 is a representation of the general structure of the hepatitis delta virus ribozyme domain known in the art.

Figure 5 is a representation of the general structure of the *Neurospora* VS RNA enzyme motif.

Figure 6 is a schematic representation of an RNase H accessibility assay. Specifically, the left side of Figure 6 is a diagram of complementary DNA oligonucleotides bound to accessible sites on the target RNA. Complementary DNA oligonucleotides are represented by broad lines labeled A, B, and C. Target RNA is represented by the thin, twisted line. The right side of Figure 5 is a schematic of a gel separation of uncut target RNA from a cleaved target RNA. Detection of target RNA is by autoradiography of body-labeled, T7 transcript. The bands common to each lane represent uncleaved target RNA; the bands unique to each lane represent the cleaved products.

Ribozymes

Ribozymes of this invention block to some extent apo(a) expression and can be used to treat disease or diagnose such disease. Ribozymes will be delivered to cells in culture and to tissues in animal models of Lp(a). Ribozyme cleavage of apo(a) mRNA in these systems may prevent or alleviate disease symptoms or conditions.

Target sites

Targets for useful ribozymes can be determined as disclosed in Draper et al supra, Sullivan *et al.*, supra, as well as by Draper et al., "Method and reagent for treatment of arthritic conditions U.S.S.N. 08/152,487, filed 11/12/93, and hereby incorporated by reference herein in totality. Rather than repeat the guidance provided in those documents here, below are provided specific examples of such methods, not limiting to those in the art. Ribozymes to such targets are designed as described in those applications and synthesized to be tested *in vitro* and *in vivo*, as also described. Such ribozymes can also be optimized and delivered as described therein. While specific examples to monkey and human RNA

are provided, those in the art will recognize that the equivalent human RNA targets described can be used as described below. Thus, the same target may be used, but binding arms suitable for targetting human RNA sequences are present in the ribozyme. Such targets may also be selected as described below.

The sequence of human and monkey apo(a) mRNA can be screened for accessible sites using a computer folding algorithm. Regions of the mRNA that did not form secondary folding structures and that contain potential hammerhead or hairpin ribozyme cleavage sites can be identified. These sites are shown in Tables II, IV, and VI - VII. (All sequences are 5' to 3' in the tables.) While monkey and human sequences can be screened and ribozymes thereafter designed, the human targetted sequences are of most utility. However, as discussed in Stinchcomb *et al.* "Method and Composition for Treatment of Restenosis and Cancer Using Ribozymes," U.S.S.N. 08/245,466, filed 5/18/94, and hereby incorporated by reference herein, monkey targeted ribozmes are useful to test efficacy of action of the ribozyme prior to testing in humans. The nucleotide base position is noted in the Tables as that site to be cleaved by the designated type of ribozyme.

Ribozyme target sites were chosen such that the cleavage sites are present in apo(a) mRNA but completely absent in the mRNA of plasminogen (Tables II, IV, VI and VII). This is because there exists extraordinary homology between apo(a) and plasminogen (see above).

It must be established that the sites predicted by the computer-based RNA folding algorithm correspond to potential cleavage sites. Hammerhead and hairpin ribozymes are designed that could bind and are individually analyzed by computer folding (Jaeger et al., 1989 Proc. Natl. Acad. Sci. USA, 86, 7706-7710) to assess whether the ribozyme sequences fold into the appropriate secondary structure. Those ribozymes with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA.

Referring to Figure 6, mRNA is screened for accessible cleavage sites by the method described generally in McSwiggen, *US Patent Application*

07/883,849 filed 5/1/92, entitled "Assay for ribozyme target site," hereby incorporated by reference herein. Briefly, DNA oligonucleotides representing potential hammerhead or hairpin ribozyme cleavage sites are synthesized. A polymerase chain reaction is used to generate a substrate for T7 RNA polymerase transcription from human or monkey apo(a) cDNA clones. Labeled RNA transcripts are synthesized *in vitro* from the two templates. The oligonucleotides and the labeled transcripts are annealed, RNaseH is added and the mixtures are incubated for the designated times at 37°C. Reactions are stopped and RNA separated on sequencing polyacrylamide gels. The percentage of the substrate cleaved is determined by autoradiographic quantitation using a phosphor imaging system. From these data, hammerhead or hairpin ribozyme sites are chosen as the most accessible.

Ribozymes of the hammerhead or hairpin motif are designed to anneal to various sites in the mRNA message. The binding arms are complementary to the target site sequences described above. The ribozymes are chemically synthesized. The method of synthesis used follows the procedure for normal RNA synthesis as described in Usman et al., 1987 J. Am. Chem. Soc., 109, 7845-7854 and in Scaringe et al., 1990 Nucleic Acids Res., 18, 5433-5441 and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. The average stepwise coupling yields were >98%. Inactive ribozymes are synthesized by substituting a U for G_5 and a U for A_{14} (numbering from Hertel et al., 1992 Nucleic Acids Res., 20, 3252). Hairpin ribozymes are synthesized in two parts and annealed to reconstruct the active ribozyme (Chowrira and Burke, 1992 Nucleic Acids Res., 20, 2835-2840). All ribozymes are modified to enhance stability by modification of five ribonucleotides at both the 5' and 3' ends with 2'-*O*-methyl groups. Ribozymes are purified by gel electrophoresis using general methods or are purified by high pressure liquid chromatography (HPLC; See Usman *et al.*, Synthesis, deprotection, analysis and purification of RNA and ribozymes, filed May, 18, 1994, U.S.S.N. 08/245,736, the totality of which is hereby incorporated herein by reference.) and were resuspended in water.

The sequences of the chemically synthesized ribozymes useful in this study are shown in Tables III, V, VI, and VII. Those in the art will recognize

that these sequences are representative only of many more such sequences where the enzymatic portion of the ribozyme (all but the binding arms) is altered to affect activity. For example, stem loop II sequence of hammerhead ribozymes listed in Tables III and V (5'-GGCCGAAAGGCC-3') can be altered (substitution, deletion and/or insertion) to contain any sequence provided a minimum of two base-paired stem structure can form. Similarly, stem-loop IV sequence of hairpin ribozymes listed in Tables VI and VII (5'-CACGUUGUG-3') can be altered (substitution, deletion and/or insertion) to contain any sequence provided, a minimum of two base-paired stem structure can form. The sequences listed in Tables III, V - VII may be formed of ribonucleotides or other nucleotides or non-nucleotides. Such ribozymes are equivalent to the ribozymes described specifically in the Tables.

Optimizing Ribozyme Activity

Ribozyme activity can be optimized as described by Stinchcomb *et al.*, supra. The details will not be repeated here, but include altering the length of the ribozyme binding arms (stems I and III, see Figure 2c), or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 Nature 344, 565; Pieken et al., 1991 Science 253, 314; Usman and Cedergren, 1992 Trends in Biochem. Sci. 17, 334; Usman et al., International Publication No. WO 93/15187; and Rossi et al., International Publication No. WO 91/03162, as well as Usman,N. et al. US Patent Application 07/829,729, and Sproat, European Patent Application 92110298.4 and U.S. Patent 5,334,711 and Jennings *et al.*, WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules. All these publications are hereby incorporated by reference herein.), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

Sullivan, et al., supra, describes the general methods for delivery of enzymatic RNA molecules . Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. The

RNA/vehicle combination is locally delivered by direct injection or by use of a catheter, infusion pump or stent. Alternative routes of delivery include, but are not limited to, intravenous injection, intramuscular injection, subcutaneous injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of ribozyme delivery and administration are provided in Sullivan, et al., supra and Draper, et al., supra which have been incorporated by reference herein.

Another means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990 Proc. Natl. Acad. Sci. U S A, 87, 6743-7; Gao and Huang 1993 Nucleic Acids Res., 21, 2867-72; Lieber et al., 1993 Methods Enzymol., 217, 47-66; Zhou et al., 1990 Mol. Cell. Biol., 10, 4529-37). Several investigators have demonstrated that ribozymes expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet et al., 1992 Antisense Res. Dev., 2, 3-15; Ojwang et al., 1992 Proc. Natl. Acad. Sci. U S A, 89, 10802-6; Chen et al., 1992 Nucleic Acids Res., 20, 4581-9; Yu et al., 1993 Proc. Natl. Acad. Sci. U S A, 90, 6340-4; L'Huillier et al., 1992 EMBO J. 11, 4411-8; Lisziewicz et al., 1993 Proc. Natl. Acad. Sci. U. S. A., 90, 8000-4). The above ribozyme transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral, Sindbis virus, Semliki forest virus vectors).

In a preferred embodiment of the invention, a transcription unit expressing a ribozyme that cleaves apo(a) RNA is inserted into a plasmid DNA vector, a retrovirus DNA viral vector, an adenovirus DNA viral vector or an adeno-associated virus vector. These and other vectors have been

used to transfer genes to live animals (for a review see Friedman, 1989 Science 244, 1275-1281; Roemer and Friedman, 1992 Eur. J. Biochem. 208, 211-225) and leads to transient or stable gene expression. The vectors are delivered as recombinant viral particles. DNA may be delivered alone or complexed with vehicles (as described for RNA above). The DNA, DNA/vehicle complexes, or the recombinant virus particles are locally administered to the site of treatment, *e.g.*, through the use of a catheter, stent or infusion pump.

Example 1: apo(a) Hammerhead ribozymes

By engineering ribozyme motifs we have designed several ribozymes directed against apo(a) mRNA sequences. These have been synthesized with modifications that improve their nuclease resistance. These ribozymes cleave apo(a) target sequences *in vitro*.

The ribozymes will be tested for function *in vivo* by exogenous delivery to cells expressing apo(a). Ribozymes are delivered by incorporation into liposomes, by complexing with cationic lipids, by microinjection, or by expression from DNA vectors. Expression of apo(a) is monitored by ELISA, by indirect immunofluoresence, and/or by FACS analysis. Levels of apo(a) mRNA are assessed by Northern analysis, RNase protection, by primer extension analysis or by quantitative RT-PCR techniques. Ribozymes that block the induction of apo(a) protein and mRNA by more than 90% are identified.

Diagnostic uses

Ribozymes of this invention may be used as diagnostic tools to examine genetic drift and mutations within diseased cells. The close relationship between ribozyme activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple ribozymes described in this invention, one may map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with ribozymes may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets may be defined as important mediators of the disease. These experiments will lead to better treatment of the

disease progression by affording the possibility of combinational therapies (e.g., multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules). Other in vitro uses of ribozymes of this invention are well known in the art, and include detection of the presence of mRNA associated with an apo(a) related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a ribozyme using standard methodology.

In a specific example, ribozymes which can cleave only wild-type or mutant forms of the target RNA are used for the assay. The first ribozyme is used to identify wild-type RNA present in the sample and the second ribozyme will be used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA will be cleaved by both ribozymes to demonstrate the relative ribozyme efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates will also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis will require two ribozymes, two substrates and one unknown sample which will be combined into six reactions. The presence of cleavage products will be determined using an RNAse protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (i.e., apo(a)) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial diagnosis. Higher mutant form to wild-type ratios will be correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

Other embodiments are within the following claims.

TABLE I

Characteristics of Ribozymes

Group I Introns
Size: ~**200** to >1000 nucleotides.
Requires a U in the target sequence immediately 5' of the cleavage site.
Binds 4-6 nucleotides at 5' side of cleavage site.
Over 75 known members of this class. Found in *Tetrahymena thermophila* rRNA, fungal mitochondria, chloroplasts, phage T4, blue-green algae, and others.

RNAseP RNA (M1 RNA)
Size: ~290 to 400 nucleotides.
RNA portion of a ribonucleoprotein enzyme. Cleaves tRNA precursors to form mature tRNA.
Roughly 10 known members of this group all are bacterial in origin.

Hammerhead Ribozyme
Size: ~**13** to 40 nucleotides.
Requires the target sequence UH immediately 5' of the cleavage site.
Binds a variable number nucleotides on both sides of the cleavage site.
14 known members of this class. Found in a number of plant pathogens (virusoids) that use RNA as the infectious agent (Figures 1 and 2)

Hairpin Ribozyme
Size: ~50 nucleotides.
Requires the target sequence GUC immediately 3' of the cleavage site.
Binds **4-6** nucleotides at 5' side of the cleavage site and a variable number to the 3' side of the cleavage site.
Only **3** known member of this class. Found in **three** plant pathogen (satellite RNAs of the tobacco ringspot virus, **arabis mosaic virus and chicory yellow mottle virus**) which uses RNA as the infectious agent (Figure 3).

Hepatitis Delta Virus (HDV) Ribozyme
Size: **50 - 60** nucleotides (at present).
Cleavage of target RNAs recently demonstrated.
Sequence requirements not fully determined.
Binding sites and structural requirements not fully determined, although no sequences 5' of cleavage site are required.
Only 1 known member of this class. Found in human HDV (Figure 4).

***Neurospora* VS RNA Ribozyme**

Size: ~144 nucleotides (at present)

Cleavage of target RNAs recently demonstrated.
Sequence requirements not fully determined.
Binding sites and structural requirements not fully determined. Only 1 known member of this class. Found in *Neurospora* VS RNA (Figure 5).

Table II:Unique Human apo(a) HH Target sequence

nt Position	HH Target Sequence	nt Position	HH Target Sequence
127	CCAGGAU U GCUACCA	11186	ACAGAAU A UUAUCCA
151	ACAGAGU U AUCGAGG	11254	UUGGUGU U AUACCAU
154	GAGUUAU C GAGGCAC	11257	GUGUUAU A CCAUGGA
199	CCAAGCU U GGUCAUC	11266	CAUGGAU C CCAAUGU
362	CAAUGCU C AGACGCA	11305	ACAAUGU C CAGUGAC
400	GACUGUU A CCCCGGU	11347	GGCUGUU U CUGAACA
408	CCCCGGU U CCAAGCC	11348	GCUGUUU C UGAACAA
409	CCCGGUU C CAAGCCU	11423	CGAGGCU C AUUCUCC
417	CAAGCCU A GAGGCUC	11427	GCUCAUU C UCCACCA
481	CCAUGGU A AUGGACA	11429	UCAUUCU C CACCACU
571	GCAUAGU C GGACCCC	11440	CACUGUU A CAGGAAG
9031	CCACGGU A AUGGACA	11653	CACAACU C CCACGGU
10207	UCCAGAU C CUGUGGC	11670	UCCCAGU U CCAAGCA
10222	AGCCCCU U AUUGUUA	11779	CACCACU A UCACAGG
10223	GCCCCUU A UUGUUAU	11797	AACAUGU C AGUCUUG
10225	CCCUUAU U GUUAUAC	11824	ACCACAU U GGCAUCG
10345	GGCUCCU U CUGAACA	11988	GUGUCCU C ACAACUC
10346	GCUCCUU C UGAACAA	12013	CCCGGUU C CAAGCAC
10532	AAGAACU A CUGCCGA	12159	CUAUGAU A CCACACU
10543	CCGAAAU C CAGAUCC	12235	UCCAGAU U CUGGGAA
10564	AGCCCCU U GGUGUUA	12236	CCAGAUU C UGGGAAA
10570	UUGGUGU U AUACAAC	12320	ACAGAAU C AGGUGUC
10622	CGAUGCU C AGAUGCA	12327	CAGGUGU C CUAGAGA
10677	CAAGCCU A GAGGCUU	12330	GUGUCCU A GAGACUC
10687	GGCUUUU U UUGAACA	12337	AGAGACU C CCACUGU
10736	UGCUACU A CCAUUAU	12374	GCUCAUU C UGAAGCA
10741	CUACCAU U AUGGACA	12453	GCACAUU C UCCACCA
10742	UACCAUU A UGGACAG	12481	GACAUGU C AAUCUUG
10792	AAGAACU U GCCAAGC	12592	AGGCCCU U GGUGUUU
10828	CCAGCAU A GUCGGAC	12650	CGAUGCU C AGACACA
10899	CUGAGAU U CGCCCUU	12974	GCAUCCU C UUCAUUU
10900	UGAGAUU C GCCCUUG	12976	AUCCUCU U CAUUUGA
10906	UCGCCCU U GGUGUUA	13119	GCACCUU A AUAUCCC
10924	CAUGGAU C CCAGUGU	13226	CUCGAAU C UCAUGUU
10976	ACAGAAU C AAGUGUC	13228	CGAAUCU C AUGUUCA
10983	CAAGUGU C CUUGCAA	13839	UGGUAUU U UUGUGUA
10986	GUGUCCU U GCAACUC	13848	UGUGUAU A AGCUUUU
11011	CCCAGAU C CAAGCAC	13930	ACUUAUU U UGAUUUG
11098	GAGUUAU C GAGGCUC	13931	CUUAUUU U GAUUUGA
11170	CUGGCAU C AGAGGAC		

Table III: Unique Human apo(a) HH Ribozyme Sequence

nt. Position	Human apo (a) HH Ribozyme Sequence
127	UGGUAGC CUGAUGAGGCCGAAAGGCCGAA AUCCUGG
151	CCUCGAU CUGAUGAGGCCGAAAGGCCGAA ACUCUGU
154	GUGCCUC CUGAUGAGGCCGAAAGGCCGAA AUAACUC
199	GAUGACC CUGAUGAGGCCGAAAGGCCGAA AGCUUGG
362	UGCGUCU CUGAUGAGGCCGAAAGGCCGAA AGCAUUG
400	ACCGGGG CUGAUGAGGCCGAAAGGCCGAA AACAGUC
408	GGCUUGG CUGAUGAGGCCGAAAGGCCGAA ACCGGGG
409	AGGCUUG CUGAUGAGGCCGAAAGGCCGAA AACCGGG
417	GAGCCUC CUGAUGAGGCCGAAAGGCCGAA AGGCUUG
481	UGUCCAU CUGAUGAGGCCGAAAGGCCGAA ACCAUGG
571	GGGGUCC CUGAUGAGGCCGAAAGGCCGAA ACUAUGC
9031	UGUCCAU CUGAUGAGGCCGAAAGGCCGAA ACCGUGG
10207	GCCACAG CUGAUGAGGCCGAAAGGCCGAA AUCUGGA
10222	UAACAAU CUGAUGAGGCCGAAAGGCCGAA AGGGGCU
10223	AUAACAA CUGAUGAGGCCGAAAGGCCGAA AAGGGGC
10225	GUAUAAC CUGAUGAGGCCGAAAGGCCGAA AUAAGGG
10345	UGUUCAG CUGAUGAGGCCGAAAGGCCGAA AGGAGCC
10346	UUGUUCA CUGAUGAGGCCGAAAGGCCGAA AAGGAGC
10532	UCGGCAG CUGAUGAGGCCGAAAGGCCGAA AGUUCUU
10543	GGAUCUG CUGAUGAGGCCGAAAGGCCGAA AUUUCGG
10564	UAACACC CUGAUGAGGCCGAAAGGCCGAA AGGGGCU
10570	GUUGUAU CUGAUGAGGCCGAAAGGCCGAA ACACCAA
10622	UGCAUCU CUGAUGAGGCCGAAAGGCCGAA AGCAUCG
10677	AAGCCUC CUGAUGAGGCCGAAAGGCCGAA AGGCUUG
10687	UGUUCAA CUGAUGAGGCCGAAAGGCCGAA AAAAGCC
10736	AUAAUGG CUGAUGAGGCCGAAAGGCCGAA AGUAGCA
10741	UGUCCAU CUGAUGAGGCCGAAAGGCCGAA AUGGUAG
10742	CUGUCCA CUGAUGAGGCCGAAAGGCCGAA AAUGGUA
10792	GCUUGGC CUGAUGAGGCCGAAAGGCCGAA AGUUCUU
10828	GUCCGAC CUGAUGAGGCCGAAAGGCCGAA AUGCUGG
10899	AAGGGCG CUGAUGAGGCCGAAAGGCCGAA AUCUCAG
10900	CAAGGGC CUGAUGAGGCCGAAAGGCCGAA AAUCUCA
10906	UAACACC CUGAUGAGGCCGAAAGGCCGAA AGGGCGA
10924	ACACUGG CUGAUGAGGCCGAAAGGCCGAA AUCCAUG
10976	GACACUU CUGAUGAGGCCGAAAGGCCGAA AUUCUGU
10983	UUGCAAG CUGAUGAGGCCGAAAGGCCGAA ACACUUG
10986	GAGUUGC CUGAUGAGGCCGAAAGGCCGAA AGGACAC
11011	GUGCUU GAUGAGGCCGAAAGGCCGAA AUCUGGG
11098	GAGCCU GAUGAGGCCGAAAGGCCG AUAACUC
11170	GUCCUC GAUGAGGCCGAAAGGCCG . AUGCCAG
11186	UGGAU CUGAUGAGGCCGAAAGGCCGAA AUUCUGU
11254	AUGGUAU CUGAUGAGGCCGAAAGGCCGAA ACACCAA
11257	UCCAUGG CUGAUGAGGCCGAAAGGCCGAA AUAACAC
11266	ACAUUGG CUGAUGAGGCCGAAAGGCCGAA AUCCAUG

11305	GUCACUG CUGAUGAGGCCGAAAGGCCGAA ACAUUGU
11347	UGUUCAG CUGAUGAGGCCGAAAGGCCGAA AACAGCC
11348	UUGUUCA CUGAUGAGGCCGAAAGGCCGAA AAACAGC
11423	GGAGAAU CUGAUGAGGCCGAAAGGCCGAA AGCCUCG
11427	UGGUGGA CUGAUGAGGCCGAAAGGCCGAA AAUGAGC
11429	AGUGGUG CUGAUGAGGCCGAAAGGCCGAA AGAAUGA
11440	CUUCCUG CUGAUGAGGCCGAAAGGCCGAA AACAGUG
11653	ACCGUGG CUGAUGAGGCCGAAAGGCCGAA AGUUGUG
11670	UGCUUGG CUGAUGAGGCCGAAAGGCCGAA ACUGGGA
11779	CCUGUGA CUGAUGAGGCCGAAAGGCCGAA AGUGGUG
11797	CAAGACU CUGAUGAGGCCGAAAGGCCGAA ACAUGUU
11824	CGAUGCC CUGAUGAGGCCGAAAGGCCGAA AUGUGGU
11988	GAGUUGU CUGAUGAGGCCGAAAGGCCGAA AGGACAC
12013	GUGCUUG CUGAUGAGGCCGAAAGGCCGAA AACCGGG
12159	AGUGUGG CUGAUGAGGCCGAAAGGCCGAA AUCAUAG
12235	UUCCCAG CUGAUGAGGCCGAAAGGCCGAA AUCUGGA
12236	UUUCCCA CUGAUGAGGCCGAAAGGCCGAA AAUCUGG
12320	GACACCU CUGAUGAGGCCGAAAGGCCGAA AUUCUGU
12327	UCUCUAG CUGAUGAGGCCGAAAGGCCGAA ACACCUG
12330	GAGUCUC CUGAUGAGGCCGAAAGGCCGAA AGGACAC
12337	ACAGUGG CUGAUGAGGCCGAAAGGCCGAA AGUCUCU
12374	UGCUUCA CUGAUGAGGCCGAAAGGCCGAA AAUGAGC
12453	UGGUGGA CUGAUGAGGCCGAAAGGCCGAA AAUGUGC
12481	CAAGAUU CUGAUGAGGCCGAAAGGCCGAA ACAUGUC
12592	AAACACC CUGAUGAGGCCGAAAGGCCGAA AGGGCCU
12650	UGUGUCU CUGAUGAGGCCGAAAGGCCGAA AGCAUCG
12974	AAAUGAA CUGAUGAGGCCGAAAGGCCGAA AGGAUGC
12976	UCAAAUG CUGAUGAGGCCGAAAGGCCGAA AGAGGAU
13119	GGGAUAU CUGAUGAGGCCGAAAGGCCGAA AAGGUGC
13226	AACAUGA CUGAUGAGGCCGAAAGGCCGAA AUUCGAG
13228	UGAACAU CUGAUGAGGCCGAAAGGCCGAA AGAUUCG
13839	UACACAA CUGAUGAGGCCGAAAGGCCGAA AAUACCA
13848	AAAAGCU CUGAUGAGGCCGAAAGGCCGAA AUACACA
13930	CAAAUCA CUGAUGAGGCCGAAAGGCCGAA AAUAAGU
13931	UCAAAUC CUGAUGAGGCCGAAAGGCCGAA AAAUAAG

Table IV: Unique Monkey apo(a) HH Target Sequence

nt. Position	HH Target Sequence	nt. Position	HH Target Sequence
127	CUGCCGU C GCaCCUC	11170	ACAaUgU C UGGugAC
151	CUGCCGU C GCaCCUC	11186	ACAGAAU C AAGUGUC
154	CUGCCgU C GcaCCUC	11254	gCUUcUU c UgaAGAA
199	CCCCGGU U CCAAGCC	11257	GACUGCU A CCAUGGU
362	AGAGGCU C CUUCCGA	11266	GAGUUAU C GAGGCUC
400	GGCUCCU U CCGAACA	11305	CGAGGCU C AUUCUCC
408	GGCUCCU U CCGAACA	11347	UCAUUCU C CACCACU
409	GGCUCCU U CCGAACA	11348	GACAUGU C AGUCUUG
417	GGCUCCU U CCGAACA	11423	UCUUGGU C CUCUAUG
481	GCUCCUU C CGAACAA	11427	UGGUCCU C UAUGACA
571	ACAGAGU U AUCGAGG	11429	UGGUCCU C UAUGACA
9031	GAGUUAU C GAGGCAc	11440	GUCCUCU A UGACACC
10207	CCACACU C uCAUAGU	11653	auAGAAU A CUACCCA
10222	CCACACU C uCAUAGU	11670	auAGA[illegible]U A CUA[illegible]CCA
10223	AGAGGCU C CUUCUGA	11779	aUGgAa[illegible] c AaGUGUC
10225	AGAGGCU c CUUCUGA	11797	CAAGUGU C CUUGCaA
[illegible]345	GUGUUAU A CAACgGA	11824	UCCC[illegible] U CCAAGCA
346	AACgGAU C CCAGUGU	11988	UcGGCA[illegible] C GGAGGAU
[illegible]532	AGaGGcU u UUCUuga	12013	UCCCAUU A cgCUAUC
10543	AGAGGCU U UUcUUGA	12159	GCUCCUU C UGAACAA
10564	GAGGCuU u UCuUgaA	12235	CCAGGAU U GCUACCA
10570	AGGCUUU U cUUGAAC	12236	CCAGGAU U GCUACCA
10622	UgCUACU a CcaUUAU	12320	gaACUGU c aGUcUuG
10677	GGCACAU A CUCCACC	12327	UCUUGGU C AUCUAUG
10687	CCACUGU u ACAGGAA	12330	UGGUCAU C UAUGAUA
10736	ccACUGU u ACAGGAA	12337	GUCAUCU A UGAUACC
10741	CCACUGU u ACAGGAA	12374	UGGUGUU A CACgACu
10742	CCACUGU u ACAGGAA	12453	AgagaCU c CCACUGU
10792	CACUGUU A CaGGaAg	12481	CUGUUGU U CCgGUUC
10828	GCAUAGU C GGA[illegible]CCC	12592	GCUCAUU C UGAAGCA
10899	GCAUAGU C [illegible]CCC	12650	UCAAUCU U GGUCAUC
10900	GCAUAGU C [illegible]CCC	12974	CCACAUU C CUGGCCC
10906	AaAaACU a [illegible]aaAu	12976	GGCAAGU C AGUCUuA
10924	CAGGAAU C CAGAUGC	13119	AgGccuU c CUUCUAC
10976	CAGGAAU C CAGAUGC	13226	AGUGUCU A GGuUGUU
10983	CAGGAAU C CAGAUGC	13228	aGuGUCU a GGuUGUu
10986	CAGGAAU C CAGAUGC	13839	UGGUAUU a UUGUGUA
11011	CAGGAAU C CAGA[illegible]	13848	UAAGCUU U UcccGUC
11098	UcGcCCU U GGU[illegible]A		

Table V: Unique Monkey apo(a) HH Ribozyme Sequence

nt. Position	Monkey HH Ribozyme Sequence
127	GAGGUGC CUGAUGAGGCCGAAAGGCCGAA ACGGCAG
151	GAGGUGC CUGAUGAGGCCGAAAGGCCGAA ACGGCAG
154	GAGGUGC CUGAUGAGGCCGAAAGGCCGAA ACGGCAG
199	GGCUUGG CUGAUGAGGCCGAAAGGCCGAA ACCGGGG
362	UCGGAAG CUGAUGAGGCCGAAAGGCCGAA AGCCUCU
400	UGUUCGG CUGAUGAGGCCGAAAGGCCGAA AGGAGCC
408	UGUUCGG CUGAUGAGGCCGAAAGGCCGAA AGGAGCC
409	UGUUCGG CUGAUGAGGCCGAAAGGCCGAA AGGAGCC
417	UGUUCGG CUGAUGAGGCCGAAAGGCCGAA AGGAGCC
481	UUGUUCG CUGAUGAGGCCGAAAGGCCGAA AAGGAGC
571	CCUCGAU CUGAUGAGGCCGAAAGGCCGAA ACUCUGU
9031	GUGCCUC CUGAUGAGGCCGAAAGGCCGAA AUAACUC
10207	ACUAUGA CUGAUGAGGCCGAAAGGCCGAA AGUGUGG
10222	ACUAUGA CUGAUGAGGCCGAAAGGCCGAA AGUGUGG
10223	UCAGAAG CUGAUGAGGCCGAAAGGCCGAA AGCCUCU
10225	UCAGAAG CUGAUGAGGCCGAAAGGCCGAA AGCCUCU
10345	UCCGUUG CUGAUGAGGCCGAAAGGCCGAA AUAACAC
10346	ACACUGG CUGAUGAGGCCGAAAGGCCGAA AUCCGUU
10532	UCAAGAA CUGAUGAGGCCGAAAGGCCGAA AGCCUCU
10543	UCAAGAA CUGAUGAGGCCGAAAGGCCGAA AGCCUCU
10564	UUCAAGA CUGAUGAGGCCGAAAGGCCGAA AAGCCUC
10570	GUUCAAG CUGAUGAGGCCGAAAGGCCGAA AAAGCCU
10622	AUAAUGG CUGAUGAGGCCGAAAGGCCGAA AGUAGCA
10677	GGUGGAG CUGAUGAGGCCGAAAGGCCGAA AUGUGCC
10687	UUCCUGU CUGAUGAGGCCGAAAGGCCGAA ACAGUGG
10736	UUCCUGU CUGAUGAGGCCGAAAGGCCGAA ACAGUGG
10741	UUCCUGU CUGAUGAGGCCGAAAGGCCGAA ACAGUGG
10742	UUCCUGU CUGAUGAGGCCGAAAGGCCGAA ACAGUGG
10792	CUUCCUG CUGAUGAGGCCGAAAGGCCGAA AACAGUG
10828	GGGGUCC CUGAUGAGGCCGAAAGGCCGAA ACUAUGC
10899	GGGGUCC CUGAUGAGGCCGAAAGGCCGAA ACUAUGC
10900	GGGGUCC CUGAUGAGGCCGAAAGGCCGAA ACUAUGC
10906	AUUUGGA CUGAUGAGGCCGAAAGGCCGAA AGUUUUU
10924	GCAUCUG CUGAUGAGGCCGAAAGGCCGAA AUUCCUG
10976	GCAUCUG CUGAUGAGGCCGAAAGGCCGAA AUUCCUG
10983	GCAUCUG CUGAUGAGGCCGAAAGGCCGAA AUUCCUG
10986	GCAUCUG CUGAUGAGGCCGAAAGGCCGAA AUUCCUG
11011	GCAUCUG CUGAUGAGGCCGAAAGGCCGAA AUUCCUG
11098	UAACACC CUGAUGAGGCCGAAAGGCCGAA AGGGCGA
11170	GUCACCA CUGAUGAGGCCGAAAGGCCGAA ACAUUGU
11186	GACACUU CUGAUGAGGCCGAAAGGCCGAA AUUCUGU
11254	UUCUUCA CUGAUGAGGCCGAAAGGCCGAA AAGAAGC
11257	ACCAUGG CUGAUGAGGCCGAAAGGCCGAA AGCAGUC
11266	GAGCCUC CUGAUGAGGCCGAAAGGCCGAA AUAACUC

11305	GGAGAAU	CUGAUGAGGCCGAAAGGCCGAA AGCCUCG
11347	AGUGGUG	CUGAUGAGGCCGAAAGGCCGAA AGAAUGA
11348	CAAGACU	CUGAUGAGGCCGAAAGGCCGAA ACAUGUC
11423	CAUAGAG	CUGAUGAGGCCGAAAGGCCGAA ACCAAGA
11427	UGUCAUA	CUGAUGAGGCCGAAAGGCCGAA AGGACCA
11429	UGUCAUA	CUGAUGAGGCCGAAAGGCCGAA AGGACCA
11440	GGUGU[illegible]	CUGAUGAGGCCGAAAGGCCGAA AGAGGAC
11653	UGGGUA[illegible]	CUGAUGAGGCCGAAAGGCCGAA AUUCUAU
11670	UGGGUAG	CUGAUGAGGCCGAAAGGCCGAA AUUCUAU
11779	GACACUU	CUGAUGAGGCCGAAAGGCCGAA AUUCCAU
11797	UUGCAAG	CUGAUGAGGCCGAAAGGCCGAA ACACUUG
11824	UGCUUGG	CUGAUGAGGCCGAAAGGCCGAA ACUGGGA
11988	AUCCUCC	CUGAUGAGGCCGAAAGGCCGAA AUGCCGA
12013	GAUAGCG	CUGAUGAGGCCGAAAGGCCGAA AAUGGGA
12159	UUGUUCA	CUGAUGAGGCCGAAAGGCCGAA AAGGACC
12235	UGGUAGC	CUGAUGAGGCCGAAAGGCCGAA AUCCUGG
12236	UGGUAGC	CUGAUGAGGCCGAAAGGCCGAA AUCCUGG
12320	CAAGACU	CUGAUGAGGCCGAAAGGCCGAA ACAGUUC
12327	CAUAGAU	CUGAUGAGGCCGAAAGGCCGAA ACCAAGA
12330	UAUCAUA	CUGAUGAGGCCGAAAGGCCGAA AUGACCA
12337	GGUAUCA	CUGAUGAGGCCGAAAGGCCGAA AGAUGAC
12374	AGUCGUG	CUGAUGAGGCCGAAAGGCCGAA AACACCA
12453	ACAGUGG	CUGAUGAGGCCGAAAGGCCGAA AGUCUCU
12481	GAACCGG	CUGAUGAGGCCGAAAGGCCGAA ACAACAG
12592	UGCUUCA	CUGAUGAGGCCGAAAGGCCGAA AAUGAGC
12650	GAUGACC	CUGAUGAGGCCGAAAGGCCGAA AGAUUGA
12974	GGGCCAG	CUGAUGAGGCCGAAAGGCCGAA AAUGUGG
12976	UAAGACU	CUGAUGAGGCCGAAAGGCCGAA ACUUGCC
13119	GUAGAAG	CUGAUGAGGCCGAAAGGCCGAA AAGGCCU
13226	AACAACC	CUGAUGAGGCCGAAAGGCCGAA AGACACU
13228	AACAACC	CUGAUGAGGCCGAAAGGCCGAA AGACACU
13839	UACACAA	CUGAUGAGGCCGAAAGGCCGAA AAUACCA
13848	GACGGGA	CUGAUGAGGCCGAAAGGCCGAA AAGCUUA

Table VI: Unique Human apo(a) Hairpin Ribozyme Sequence

nt. position	Hairpin Ribozyme Sequence	Substrate Sequence
378	GGCGCGAC AGAA GUCC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GGACU GCC GUCGCGCC
381	GGAGGCGC AGAA GCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CUGCC GUC GCGCCUCC
440	UUUGCUCA AGAA GUGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GCACC GAC UGAGCAAA
7964	UCUGCUCA AGAA GUGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GCACC GAC UGAGCAGA
10215	CAAUAAGG AGAA GCCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	UGGCA GCC CCUUAUUG
10534	UGGAUUUC AGAA GUAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CUACU GCC GAAAUCCA
10557	CACCAAGG AGAA GCCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	UGGCA GCC CCUUGGUG
10638	GGGACGAA AGAA GUCC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GGACU GCC UUCGUCCC
10700	UUUCCUCA AGAA GUGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GCACU GAC UGAGGAAA
11343	UGUUCAGA AGAA GCCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CGGCU GUU UCUGAACA
11379	CAGUCCUG AGAA GUGG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CCACA GUC CAGGACUG
12342	ACUGGAAC AGAA GUGG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CCACU GUU GUUCCAGU
12804	GGCUCCUG AGAA GCCC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GGGCU GCC CAGGAGCC
12877	AGGGUUAC AGAA GUAA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	UUACU GCC GUAACCCU
13139	GAGCAGCA AGAA GCAC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GUGCU GAC UGCUGCUC
13256	GCUCCAAG AGAA GCCU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	AGGCU GUU CUUGGAGC
13522	ACCCUGGC AGAA GUCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	UGACA GUU GCCAGGGU
13794	UAGCUGGG AGAA GUGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	ACACU GUU CCCAGCUA

Table VII: Unique Monkey apo(a) Hairpin Ribozyme Sequence

nt. Position	Hairpin Ribozyme Sequence	Substrate Sequence
57	GGUGCGAC AGAA GUCC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GGACU GCC GUCGCACC
60	GGAGGUGC AGAA GCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CUGCC GUC GCACCUCC
119	UUUGCUCA AGAA GUGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GCACC GAC UGAGCAAA
318	CAAUAAGG AGAA GCCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	UGGCA GCC CCUUAUUG
660	CAAUAAGG AGAA GCCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	UGGCA GCC CCUUAUUG
744	GGAGGUGC AGAA GCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CUGCA GUC GCACCUCC
803	UUUGCUCA AGAA GUGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GCACC GAC UGAGCAAA
1002	CAAUAAGG AGAA GCCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	UGGCA GCC CCUUAUUG
1083	GGUGCGAC AGAA GUCC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GGACU GCC GUCGCACC
1086	GGAGGUGC AGAA GCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CUGCC GUC GCACCUCC
1321	UGGAUUUC AGAA GUAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CUACU GCC GAAAUCCA
1344	CACCAAGG AGAA GCCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	UGGCA GCC CCUUGGUG
2130	UGUUCAGA AGAA GCCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	UGGCU GUU UCUGAACA
2500	GACCCCAG AGAA GUUU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	AAACA GCC CUGGGGUC
3129	ACCGGAAC AGAA GUGG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CCACU GUU GUUCCGGU
3683	AAGCAGCA AGAA GCAC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	GUGCU GAC UGCUGCUU
3890	AAUUUGGA AGAA GCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	CUGCC GUC UCCAAAUU
3912	UCAGUCCA AGAA GUGA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	UCACC GCC UGGACUGA
4165	UGCUGGG AGAA GUGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	ACACU GUC CCCAGCUA

Claims

1. An enzymatic RNA molecule which cleaves apo(a) mRNA .

2. An enzymatic RNA molecule of claim 1, the binding arms of which contain sequences complementary to any one of the sequences defined in any of those in Table II.

3. The enzymatic RNA molecule of claim 1, the binding arms of which contain sequences complementary to the sequences defined in any one of Tables IV, VI and VII

4. The enzymatic RNA molecule of claims 1, 2, or 3, wherein said RNA molecule is in a hammerhead motif.

5. The enzymatic RNA molecule of claim 1, 2, or 3, wherein said RNA molecule is in a hairpin, hepatitis delta virus, group 1 intron, *Neurospora* VS RNA or RNaseP RNA motif.

6. The enzymatic RNA molecule of claim 5, wherein said ribozyme comprises between 12 and 100 bases complementary to said mRNA.

7. The enzymatic RNA molecule of claim 6, wherein said ribozyme comprises between 14 and 24 bases complementary to said mRNA.

8. Enzymatic RNA molecule consisting essentially of any sequence selected from the group of those shown in Tables III, V, VI, and VII.

9. A mammalian cell including an enzymatic RNA molecule of claims 1, 2, or 3.

10. The cell of claim 8, wherein said cell is a human cell.

11. An expression vector including nucleic acid encoding an enzymatic RNA molecule or multiple enzymatic molecules of claims 1, 2, or 3 in a manner which allows expression of that enzymatic RNA molecule(s) within a mammalian cell.

12. A mammalian cell including an expression vector of claim 11.

13. The cell of claim 13, wherein said cell is a human cell.

14. A method for treatment of a condition related to elevated plasma Lp(a) levels by administering to a patient an enzymatic nucleic acid molecule of claims 1, 2, or 3,

15. A method for treatment of a condition related to elevated plasma Lp(a) levels by administering to a patient an expression vector of claim 11.

16. The method of claims 14 or 15, wherein said patient is a human.

17. The method of claim 15 wherein said condition is selected from the group consisting of atherosclerosis, myocardial infraction, stroke, restenosis, and heart diseases.

18. The method of claim 17 wherein said condition is restenosis.

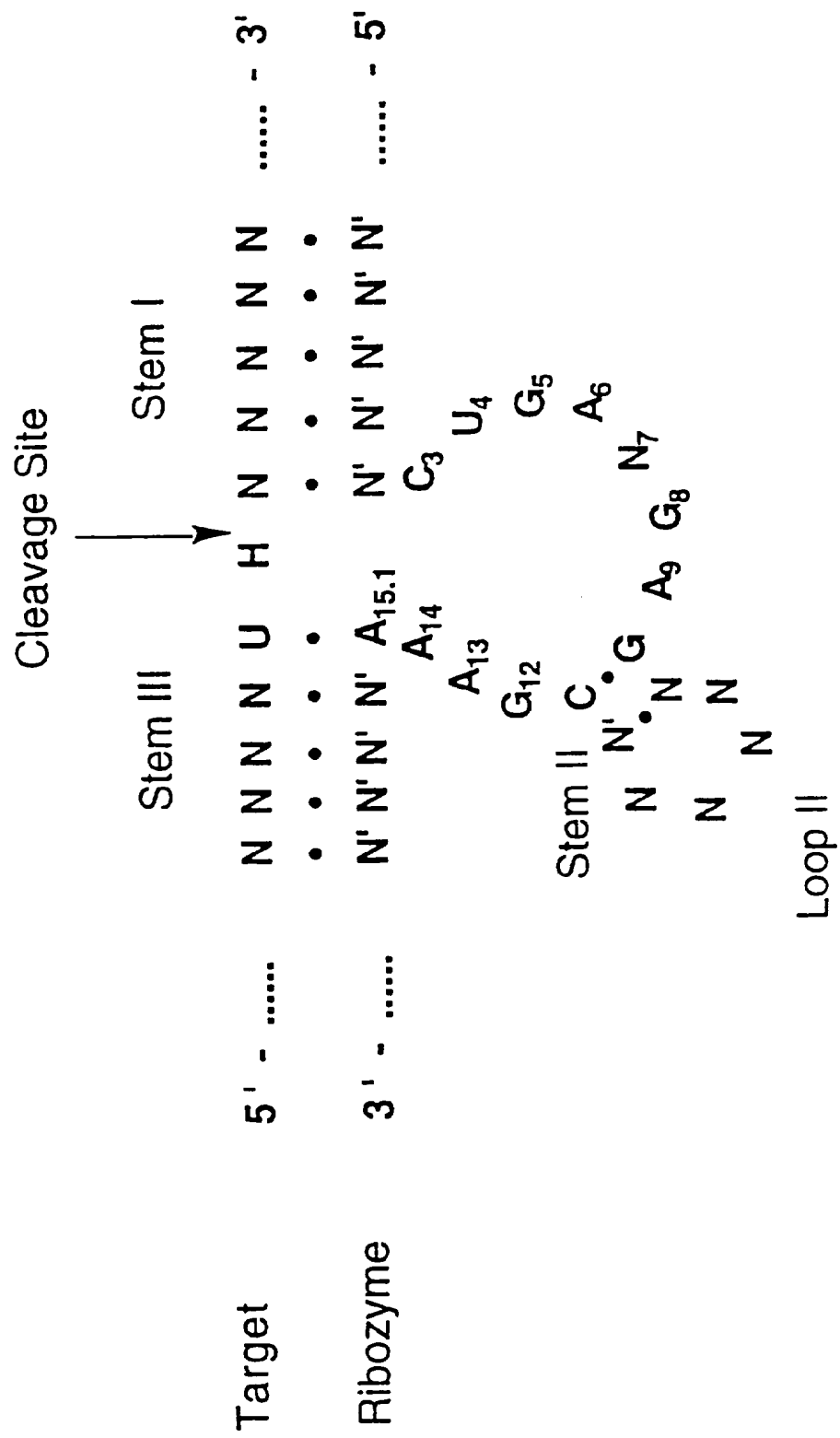
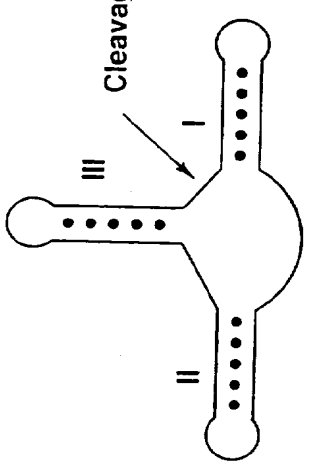

Cleavage Site
Stem I
Stem III
Stem II
Loop II
Target
Ribozyme
5' - .....
3' - .....
N N N N U H N N N N N ..... - 3'
• • • • • • • • • • ..... - 5'
N' N' N' N' A15.1 N' N' N' N'
A14
A13
G12
C3
U4
G5
A6
N7
G8
A9
C
N'
N
N
N
N
N
G

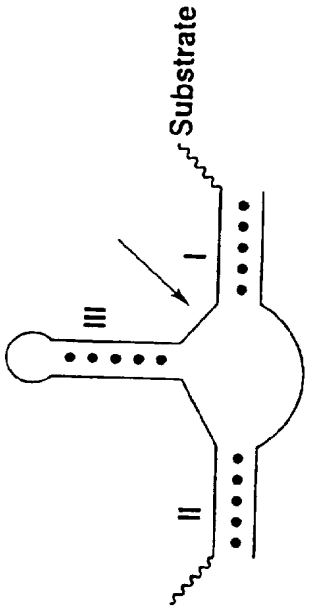
FIG. 1.

FIG. 2a.



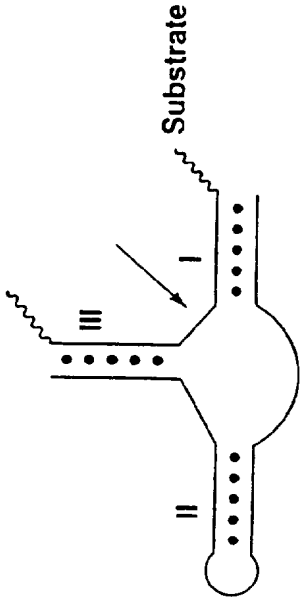
a

FIG. 2b.



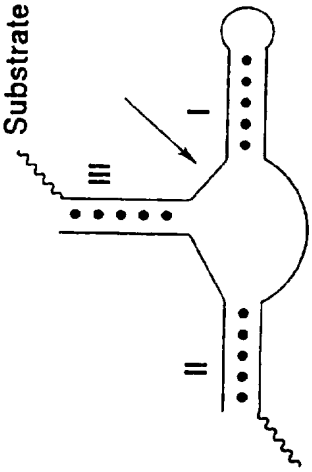
b

FIG. 2c.



c

FIG. 2d.



d

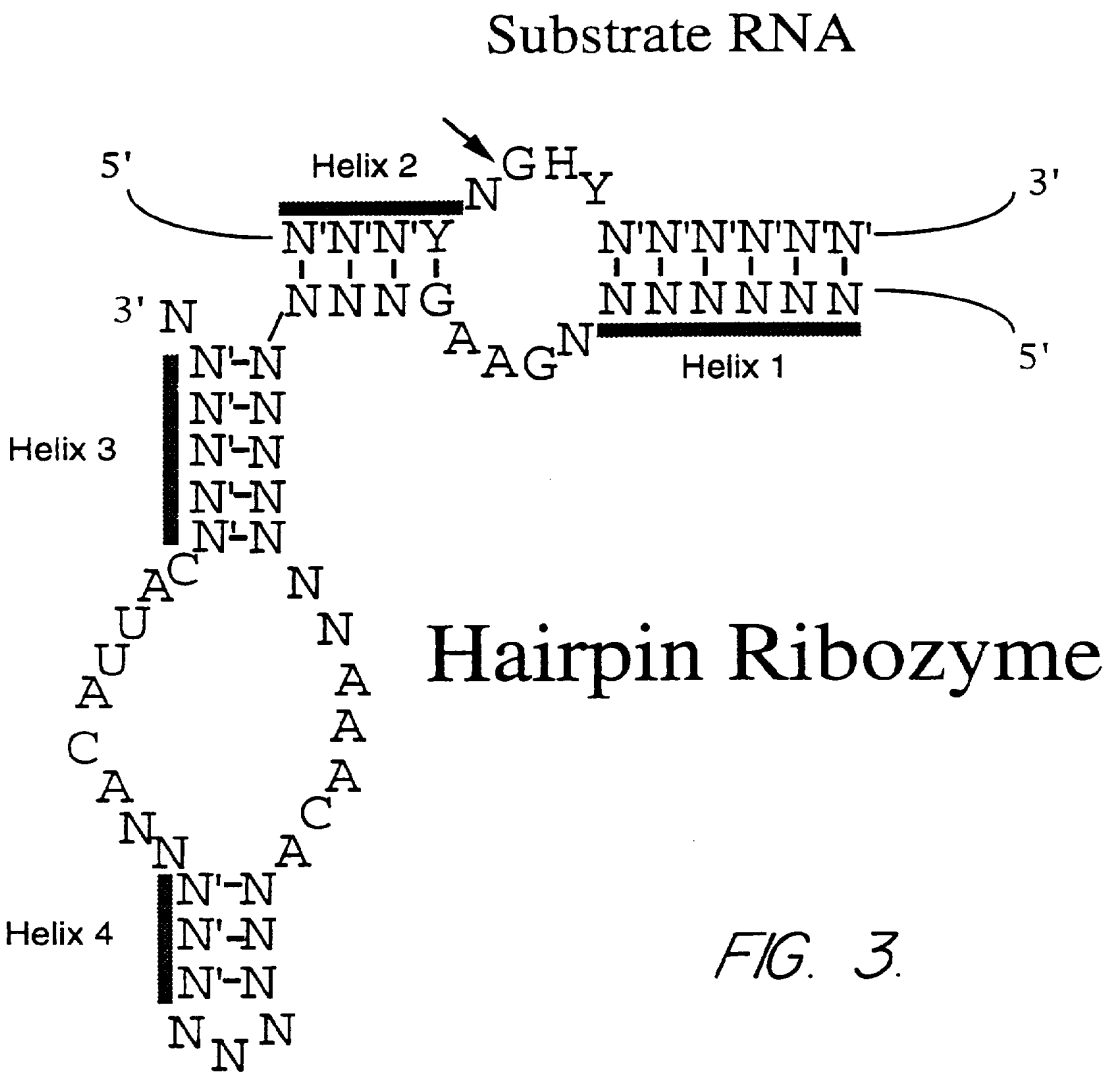

Substrate RNA
5'
Helix 2
3'
N'N'N'Y
N'N'N'N'N'N'
NNNG
NNNNNN
3' N
AAG
N
Helix 1
5'
N'-N
Helix 3
Hairpin Ribozyme
Helix 4

FIG. 3.

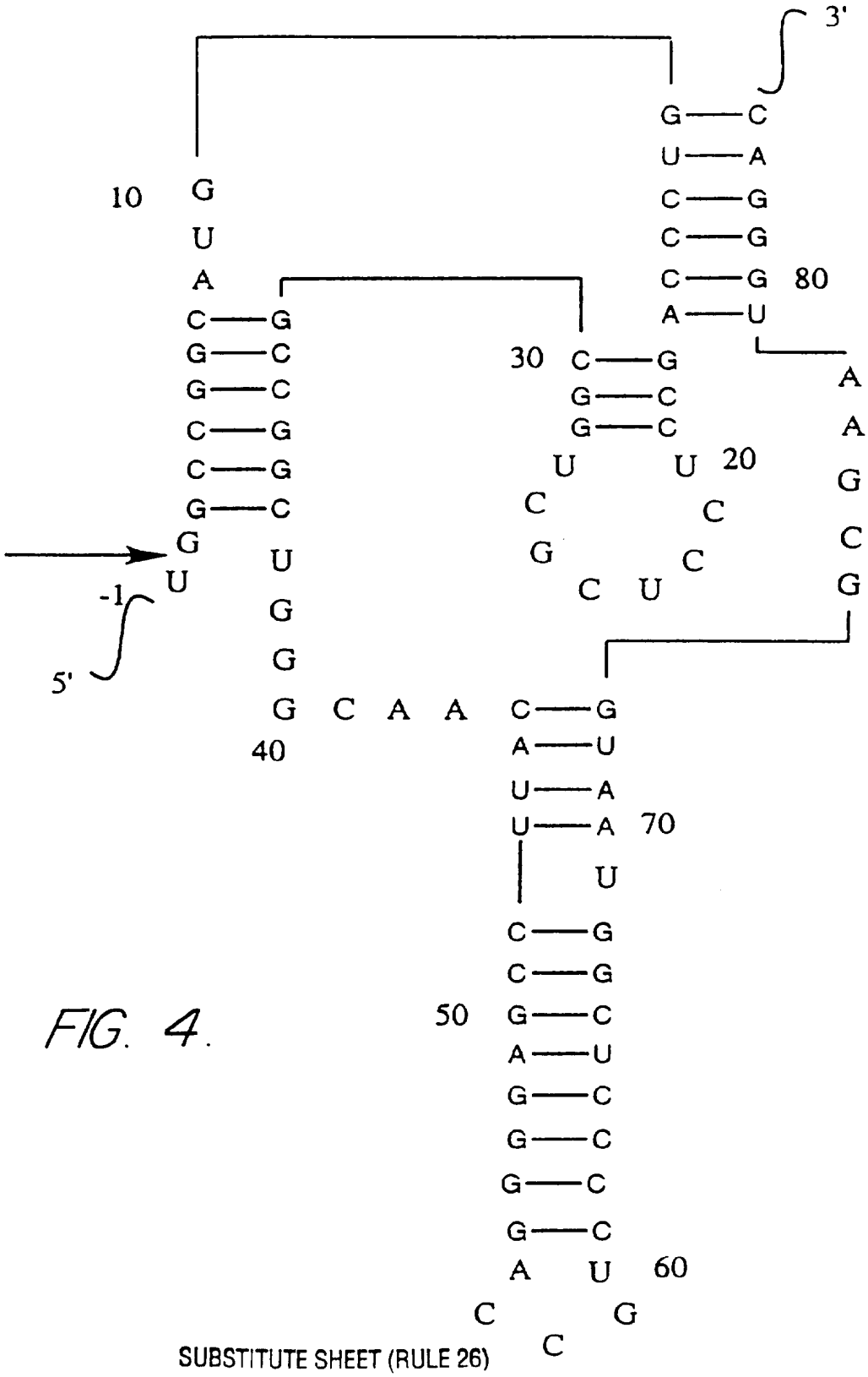


FIG. 4.

FIG. 5.

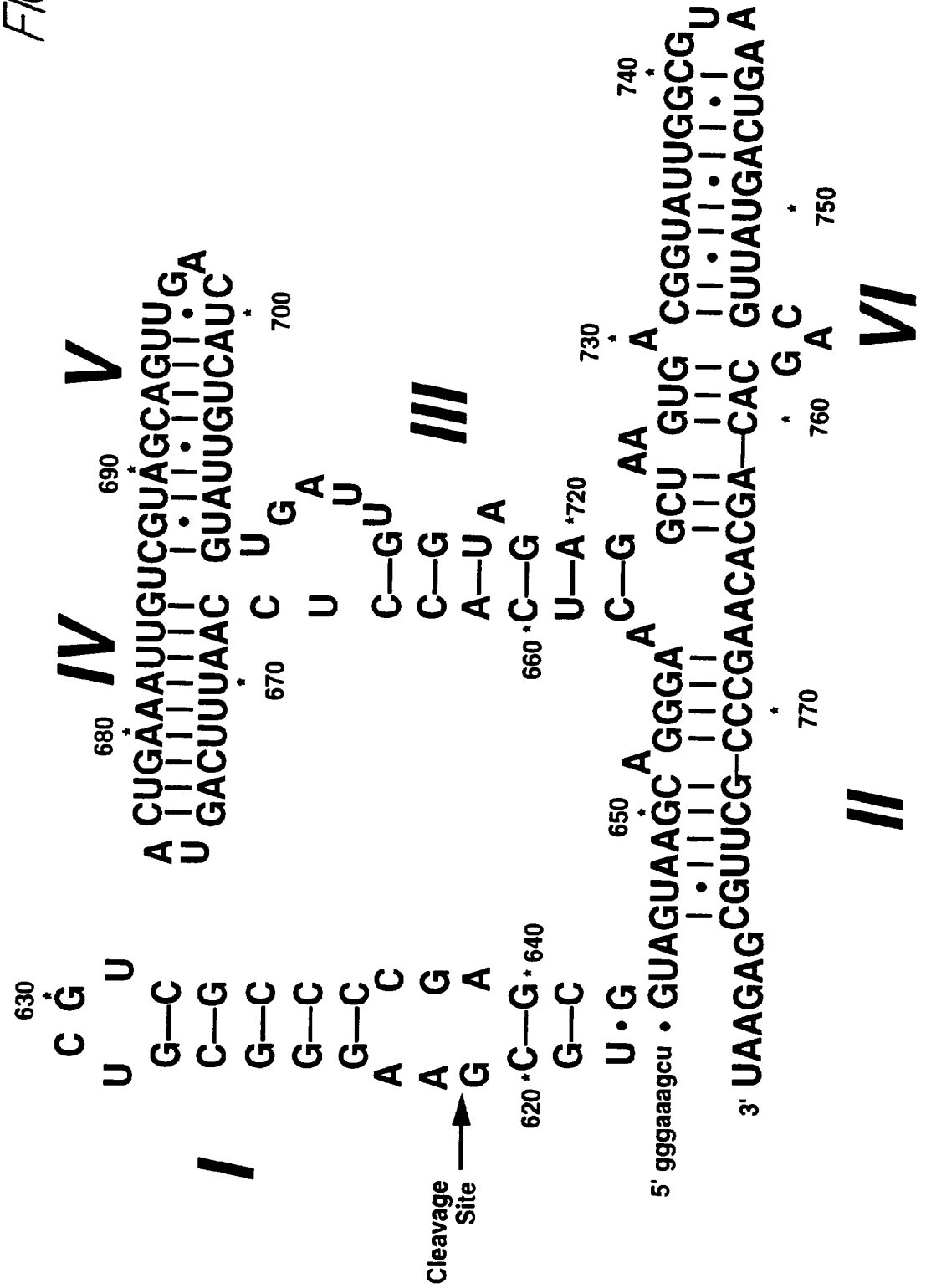
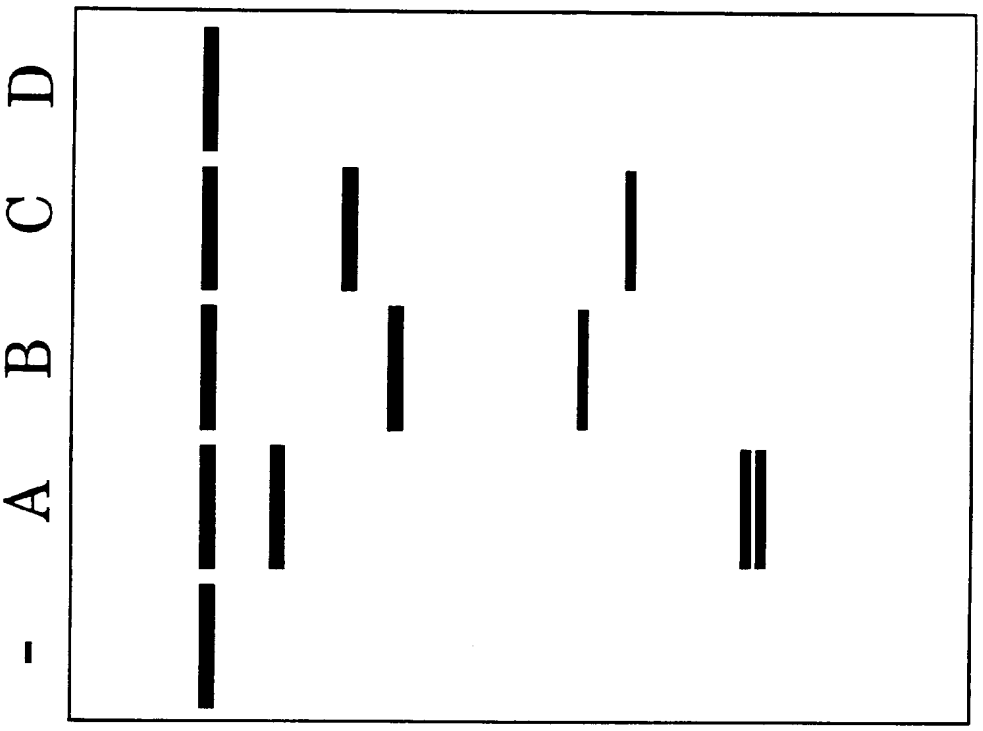
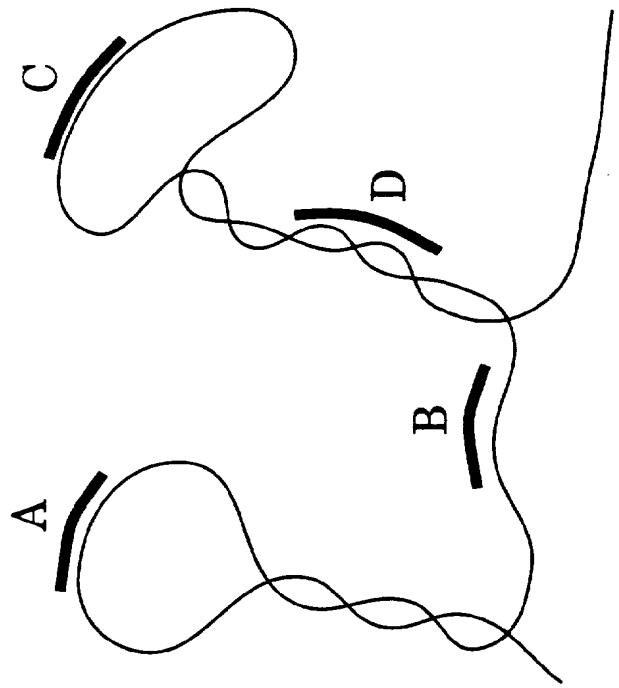

I
IV
V
III
II
VI
Cleavage Site
5' gggaaagcu
3' UAAGAG
630
640
650
660
670
680
690
700
720
730
740
750
760
770

FIG. 6.





- Body-labeled transcript (not purified)
- DNA oligo (10 nM, 100 nM and 1000 nM)
- RNAse H (0.08 -1.0 u/µl)
- 37°C, 10 min

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 95/11995

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N9/00 A61K31/70 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol. 330, 12 November 1987 LONDON GB, pages 132-137, MCLEAN, J. ET AL. 'cDNA sequence of human apolipoprotein(a) ishomologous to plasminogen' cited in the application see figure 1 ---	1-3,8, 14-18
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, 5 April 1989 MD US, pages 5957-5965, TOMLINSON, J. ET AL. 'Rhesus monkey apolipoprotein(a)' cited in the application see figure 1 --- -/--	1-3,8, 14-18

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
28 February 1996	06. 03. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 95/11995

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CURRENT OPINION IN STRUCTURAL BIOLOGY, vol. 4, June 1994 LONDON GB, pages 322-330, XP 000523747 SYMONS, R.H. 'Ribozymes' see the whole document ---	1-18
A	CIRCULATION, vol. 88, no. 3, September 1993 pages 935-940, MOLITERNO, D. ET AL. 'Relation of plasma lipoprotein(a) to infarct artery patency in survivors of myocardial infarction' cited in the application see the whole document -----	14-18

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